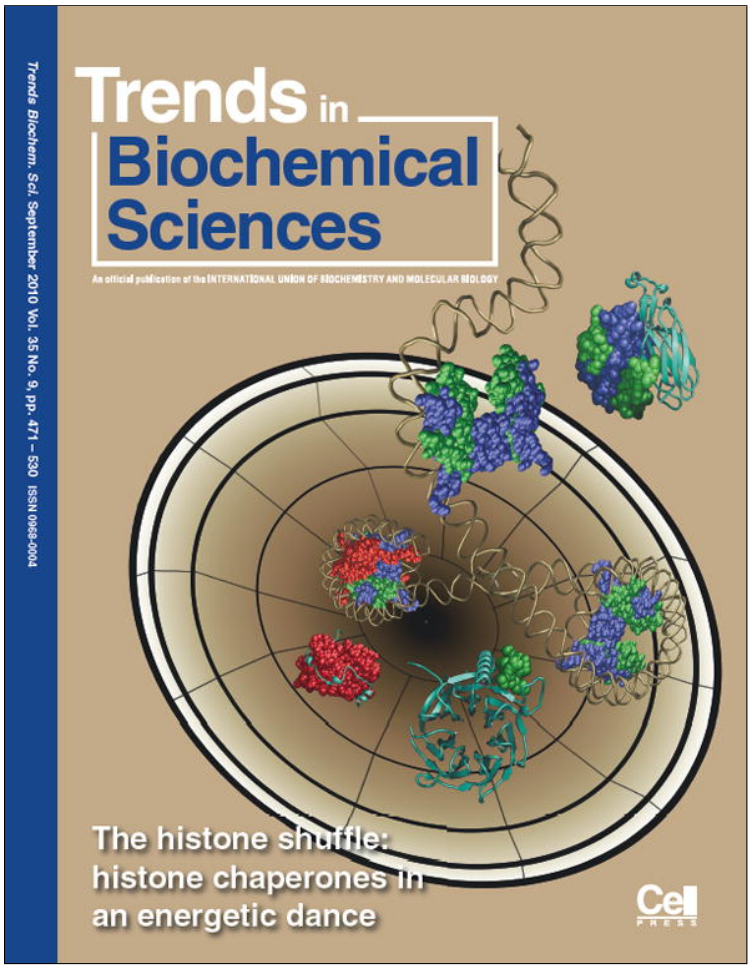

Review

The carboxy terminal domain of RNA polymerase II and alternative splicing

Manuel J. Muñoz, Manuel de la Mata[a] and Alberto R. Kornblihtt

Laboratorio de Fisiología y Biología Molecular, Departamento de Fisiología, Biología Molecular y Celular, IFIBYNE-CONICET, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires. Ciudad Universitaria, Pabellón 2, (C1428EHA) Buenos Aires, Argentina

Alternative splicing is controlled by *cis*-regulatory sequences present in the pre-mRNA and their cognate *trans*-acting factors, as well as by its coupling to RNA polymerase II (pol II) transcription. A unique feature of this polymerase is the presence of a highly repetitive carboxy terminal domain (CTD), which is subject to multiple regulatory post-translational modifications. CTD phosphorylation events affect the transcriptional properties of pol II and the outcome of co-transcriptional alternative splicing by mediating the effects of splicing factors and by modulating transcription elongation rates. Here, we discuss various examples of involvement of the CTD in alternative splicing regulation as well as the current methodological limitations in deciphering the detailed mechanisms of this process.

A unique domain in the polymerase that makes mRNA

The multi-subunit enzyme that transcribes protein-coding genes, RNA polymerase II (pol II), has a unique characteristic among DNA-dependent RNA polymerases: the presence of a repetitive carboxyl terminal domain (CTD) in its largest subunit, RPB1 [1]. In mammals, the CTD comprises 52 tandemly repeated heptapeptides with the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser ($Y_1S_2P_3T_4S_5P_6S_7$). The degree of adjustment to the consensus of each heptad varies with its location along the CTD (Figure 1). Soon after it became clear that the catalytic activity of RPB1 was not located in the CTD, Corden and co-workers performed a deletion analysis to dissect its function. By inhibiting the endogenous pol II with α-amanitin and transiently transfecting vectors expressing α-amanitin-resistant pol II with a truncated CTD, they discovered that the CTD was necessary for transcriptional activation by almost all transcriptional enhancers tested [2]. Pol II-mediated transcription is not sufficient to synthesize an mRNA suitable for nuclear export and translation, and must be followed by covalent modification of the nascent pre-mRNA, including capping, splicing and 3' end processing (cleavage and polyadenylation). In 1997, David Bentley's group found that the roles of the CTD were not limited to transcriptional activation, but instead involved all three pre-mRNA processing reactions and introduced the concept of an "mRNA factory" lying within the nucleus which is governed by the CTD [3]. Using Corden's strategy, they demonstrated that the CTD was affecting transcription, as well as capping, splicing and 3'end processing [3,4]. The idea of a mRNA factory containing pol II and processing factors gave strong support to the concept of coupling of transcription and pre-mRNA processing in which both processes can influence each other and occur in a highly coordinated manner within the cell nucleus. In those early days in the emerging field of coupling, our group added another level of complexity by showing that promoter identity affects alternative splicing (AS) decisions, using as a model the extra domain I (EDI) alternative exon of the human fibronectin gene [5]. Proper regulation of AS is crucial for metazoan life because: (i) AS is a major contributor in achieving a vast proteomic complexity with a limited number of genes; indeed, it was reported to affect the expression from 65% [6] to up to 90% of human genes [7,8]; (ii) mutations that either create or abolish AS regulatory sequences, also known as splicing enhancers and silencers, are a widespread source of human disease [9–13]; (iii) AS factors can be misregulated in cancer [14–17]; and (iv) AS factors have key roles in tissue development [18–20]. Interestingly, the quantity of known splicing regulators, or even RNA binding proteins in general (around 50 and 300, respectively), estimated from human genome analysis, is insufficient to explain the regulation of known AS events [21]. Nevertheless, the fact that transcription affects AS patterns allows the cell to combine two major mechanisms to tightly control its expression profiles. In this review we focus on the different ways in which mammalian transcription affects AS and how the CTD of pol II is involved in the coupling of both processes.

The CTD is target of multiple post-translational modifications

The unusual consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser contains many potential phosphorylation sites. At first glance, five out of seven residues could be phosphorylated within the consensus heptad but, in addition, proline isomerization [22,23] as well as serine and threonine glycosylation [24], have been reported as plausible post-translational modifications (PTMs) of consensus CTD residues. Non-consensus residues, such as lysine and arginine, are also present in the CTD and they can potentially be modified by acetylation, ubiquitylation, SUMOylation (lysine residues) and methylation (lysine and arginine residues). The combinatorial possibilities of the different PTMs along the 52 CTD heptads is immense, and only a fraction has been demonstrated to influence interactions

Corresponding author: Kornblihtt, A.R. (ark@fbmc.fcen.uba.ar)
[a] Present address: Friedrich Miescher Institute, Basel, Switzerland..

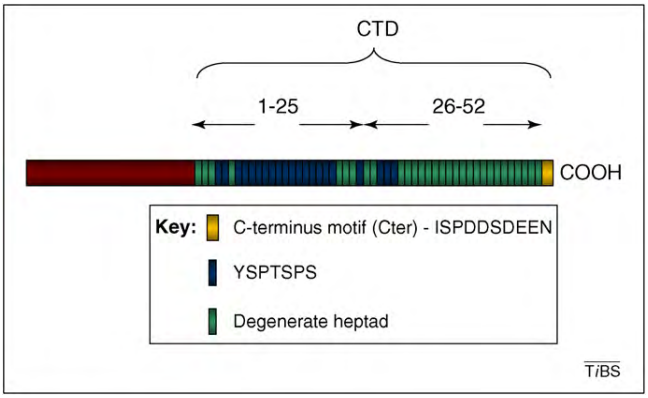

Figure 1. Repetitive structure of the CTD of the large subunit (RPB1) of RNA polymerase II. The two halves of the CTD differ in heptad composition. Repeats 1–25 are richer in the consensus sequence YSPTSPS whereas repeats 26–52 are more degenerate. The non-repetitive C-terminal motif ISPDDSDEEN is necessary to prevent full CTD degradation *in vivo*.

with a plethora of proteins, which in turn, affect many steps in the flow from genes to translatable mRNAs (for reviews see Refs [25–27]).

The CTD is necessary to transcribe endogenous genes

The experimental strategies used to identify CTD-associated functions include transient transfection of mammalian cells with vectors expressing α-amanitin-resistant pol IIs bearing wild type (WT), mutant or partially/totally truncated CTDs, together with reporter gene constructs [2–4]. Unlike endogenous genes, the chromatin of these transiently expressed reporters is not physiologically assembled, which seems to be the reason why these reporters are effectively transcribed by CTD-less pol IIs under certain circumstances, such as when transcription is controlled by weak activators, like SP1, but not by strong acidic activators, such as VP16 [2,28]. However, the requirement for the CTD seems to be absolute for the transcription of endogenous genes, as evidenced by Eick and co-workers [29], who demonstrated that a negligible number of the 1176 genes analyzed in an array were transcribed by a mutant polymerase lacking the CTD. Consistently, cell lines stably transfected with the CTD-less α-amanitin-resistant polymerase die a few days after α-amanitin is added to the cell culture [29]. The CTD interacts with chromatin remodeling and modifier factors, such as the histone methyltransferases Set1 [30] and Set2 [31] or the histone acetyltransferases p300 and PCAF (p300/CBP-associated factor) [32], which probably explains the global transcriptional defect of endogenous genes in the absence of a functional CTD. These are important considerations to take into account when assessing putative roles of the CTD in post-transcriptional events. It is important to note, however, that although the CTD is involved in many post- and co-transcriptional processing events, the effects of CTD truncation on endogenous pre-mRNA processing are difficult to study because little transcription takes place in the absence of the CTD.

CTD and pre-mRNA processing

Among the many pre-mRNA processing reactions in which the CTD participates, the CTD requirement for efficient pre-mRNA capping is probably the best characterized. The capping enzymes interact specifically with the CTD phosphorylated at Ser5 [33,34], a PTM associated with transcriptional initiation predominantly found in pol II molecules located towards the 5' end of genes [35]. Cleavage/polyadenylation factors also interact with the CTD, but when phosphorylated at Ser2 [30,36,37], a PTM that is preferentially found in pol II molecules mapping intragenically towards the 3' end of genes [35]; this modification is associated with productive transcriptional elongation. Whether the Ser5 and Ser2 of all or just some repeats need to be phosphorylated to enhance capping and cleavage/polyadenylation respectively is not known (see below). Fragmentary evidence also points to a role of the CTD in constitutive splicing as (i) transcriptional activation of pol II genes induces the association of splicing factors to sites of transcription only when pol II has a full CTD [38]; (ii) deletion of the CTD inhibits splicing of the β-globin gene [3]; and (iii) isolated CTD fragments [39], as well as purified phosphorylated pol II [40], are able to activate splicing *in vitro*. Interestingly, isolated CTD fragments cannot duplicate the effect of pol II unless the pre-mRNA is recognized via exon definition, i.e. it contains at least one complete internal exon with 3' and 5' splice sites. It is worth noting, however, that the CTD requirement for constitutive splicing appears to be specific to some exons [28]. Together with the fact that many, but not all, introns are removed co-transcriptionally [41], we might conclude that the effect of the CTD on constitutive splicing is not general and could be restricted to co-transcriptional splicing events governed by exon definition. Such a view agrees with findings that the CTD is dispensable for post-transcriptional splicing (i.e. uncoupled from transcription), as observed when pre-mRNA is injected directly into *Xenopus laevis* oocytes [42]. In any case, the CTD, conveniently located next to the pol II RNA exit channel [43], cannot act on RNA processing by itself *in vivo*: T7 RNA polymerase or RNA pol III molecules engineered to carry a pol II CTD failed to support efficient pre-mRNA processing [44].

Co-transcriptionality and coupling

In order to catalyze intron removal and exon ligation from the pre-mRNA, the spliceosome must first recognize and assemble onto specific sequences: the 5' and 3' splice sites (ss). The control of splice site recognition by the spliceosome represents the best-studied mechanism of AS regulation; indeed, many AS factors affect 5' ss recognition by U1 snRNP (small nuclear ribonucleoprotein) or 3' ss recognition by U2 snRNP. For instance, the serine/arginine-rich (SR) protein family members can influence splice site recognition by binding to the pre-mRNA and affecting spliceosome assembly directly [45]. Since the discovery that transcription influences AS [5,46], a wide range of transcriptional modulators, including pol II itself, appeared as potential AS regulators. In this scenario, co-transcriptional splicing or co-transcriptional commitment to splicing seem to be reasonable pre-requisites for coupling, i.e. for the existence of functional interactions between the transcription and splicing machineries. Nevertheless, the existence of co-transcriptionality *per se* does not necessarily imply coupling [47]. Yet, current

evidence in mammalian and yeast cells supports the notion that co-transcriptional splicing or co-transcriptional spliceosomal assembly is the rule rather than the exception [48–53]. Moreover, although splicing can occur independently of transcription *in vitro*, the process is less efficient than that *in vivo* [42,54] or in transcription-coupled *in vitro* systems [48]; these findings are consistent with the existence of coupling. In fact, co-transcriptional splicing helps to prevent pre-mRNA degradation [55] and drives pre-mRNA to associate with splicing regulatory factors such as SR proteins [56]. Rapid association of spliceosome components [57–59] and splicing regulatory factors to the nascent pre-mRNA is important to improve splicing efficiency, and to prevent back-hybridization of the nascent pre-mRNA to the DNA template strand, a process that would favor genome instability due to the accumulation of DNA breaks triggered by the single strand status of the non-template strand [60,61]. Together, the available data suggest that the evolutionary advantage of co-transcriptionality resides in allowing for coupling.

"Coupling" two modes of coupling

Two non-mutually exclusive models have been proposed to explain the coupling between transcription and AS. The recruitment coupling model involves the association of splicing factors to the transcribing polymerase complex, whereas the kinetic coupling model involves modulation of the elongation rates of pol II, which affects the timing of the window opportunity for the use of weak splice sites located upstream of stronger splice sites. In both scenarios, the CTD plays a central role: the action of specific splicing factors, as well as the modulation of the elongation rates of pol II, depend on the presence or covalent modifications of the CTD. The recruitment model is supported by findings that show that the inhibitory action of the SR protein, SRp20, on the inclusion of the fibronectin alternative exon EDI into the mRNA is CTD-dependent [28]. The involvement of the CTD was demonstrated by the use of a pol II mutant lacking the CTD (Δ0). Transient transfection experiments using EDI AS reporter minigenes showed that when transcription is carried out by the Δ0 mutant polymerase, the inclusion of the EDI alternative exon is increased when compared to a WT pol II enzyme [28]. This increase was attributed to the inability of SRp20 to exert its inhibitory effect in the absence of the CTD (Figure 2). Contradictory evidence exists regarding a physical interaction between SRp20 and pol II. Although a proteomic analysis revealed that SRp20 co-immunoprecipitates with pol II [56], it is not certain whether such an interaction might be mediated by the nascent pre-mRNA, and therefore is not direct. Evidence against the interaction, even in the presence of RNA, has been provided [59]. The existence of direct interactions between splicing factors and the pol II CTD is, in general, not as clear as it is for factors involved in capping or 3' end processing. In fact, it has been proposed that none of the SR proteins can contact the CTD strongly and directly; nevertheless, and in a yet unidentified mechanism, the CTD is required for the action of SRp20 on AS. The basic idea surrounding the recruitment model of coupling is that through dynamic association with the transcribing polymerase, the local concentration of a given splicing regulator is increased in the vicinity of pre-mRNA, thus affecting AS patterns.

The mechanism by which elongation rates affect AS patterns (kinetic coupling) can also be illustrated using the fibronectin EDI exon. EDI exon skipping occurs because the 3' ss of the upstream intron is weaker than the 3' ss of the downstream intron. A rapidly elongating transcription complex will transcribe both introns before the 5' ss of the upstream intron can be used. As a result, the 5' ss will be preferentially spliced to the strong downstream 3' ss, rather than the weak upstream 3' ss, resulting in exon skipping. However if pol II elongation is reduced by different means, the inclusion of EDI is increased significantly

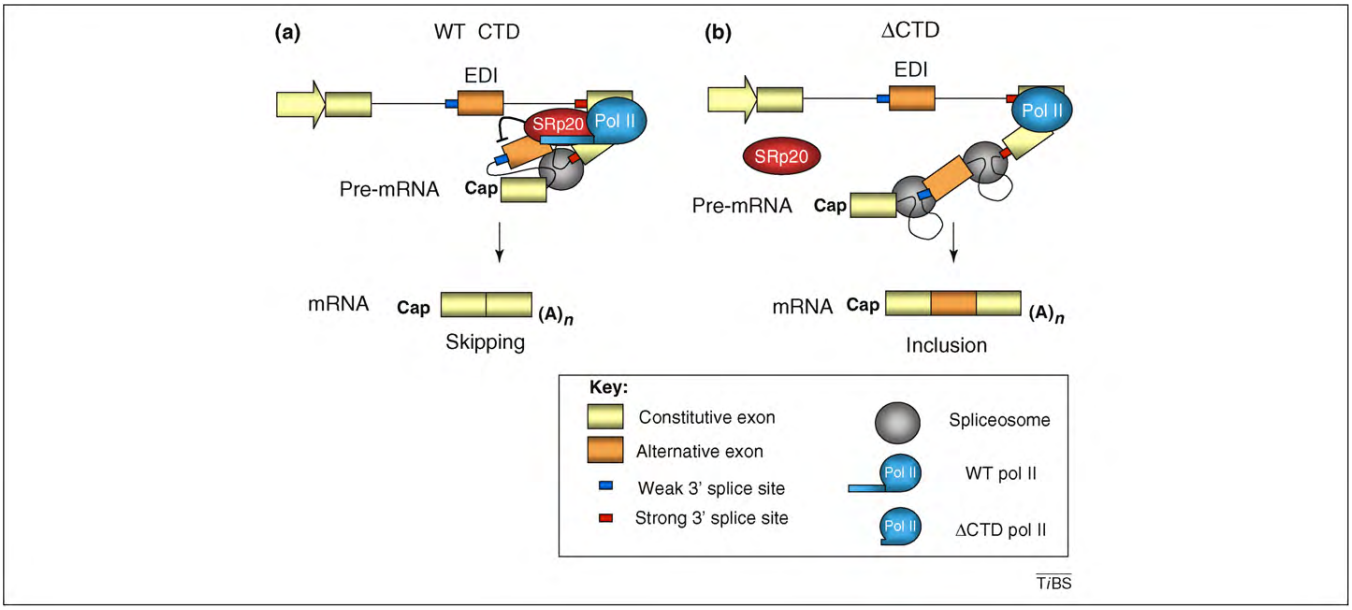

Figure 2. Recruitment coupling. The CTD of RNA polymerase II mediates the inhibitory effect of the SR protein SRp20 on the inclusion of the alternatively spliced fibronectin EDI exon. **(a)** Transcription by a WT pol II allows recruitment of SRp20 to the transcription machinery that stimulates EDI skipping. **(b)** Transcription by a mutated pol II lacking the CTD (ΔCTD) causes higher EDI inclusion because SRp20 is not recruited. Based on de la Mata *et al.* (2006) [28].

[62,63]. One possible explanation for this phenomenon would be that if the polymerase pauses between these two splice sites, the upstream intron would be spliced before the downstream intron, following a "first come first serve" pathway. Once the transcription complex resumes elongation, the downstream intron would be subsequently removed and the exon would be included. However, our laboratory recently showed that the order of intron removal of EDI flanking introns is unaffected upon changes in pol II elongation, suggesting that this regulation takes place at a very early stage during spliceosome assembly [64]. For instance, a reduced pol II elongation rate would favor an early recruitment of splicing factors to the weak splice sites regardless of the final order of intron removal (Figure 3). When a weak 3' ss is followed by a strong one, as is the case in many AS events, the transcription elongation rate can affect the relative amounts of the mRNA isoforms. However, when two consecutive strong 3' ss occur, as in constitutive splicing, transcription elongation rates are less relevant. The most direct evidence supporting this model (for a review see Ref. [65]) was obtained using a mutant form of pol II (termed C4) with a lower elongation rate [62]. In experiments using α-amanitin to inhibit endogenous pol II, the slow polymerase, in comparison to the WT enzyme, was able to increase EDI inclusion, thus confirming the inverse correlation between elongation rate and inclusion of this alternative exon. Most importantly, and of physiological relevance, *Drosophila melanogaster* carrying the C4 mutation show changes in the AS profile of transcripts encoded by the endogenous large ultrabithorax (*Ubx*) gene [62]. Interestingly, C4 heterozygous flies display a phenotype, known as the Ubx effect, where the halteres present a morphology that resembles the abnormal second pair of wings characteristic of the Ultrabithorax mutation, suggesting that the control of elongation might impact on AS, generating splicing variants that could affect organismal phenotypes.

It is easy to envision the fusion of the kinetic and recruitment models of coupling into one reality: the recruitment of specific factors might modulate the pol II elongation rate throughout the entire gene or in a specific zone. In an elegant study, Batsché and co-workers showed that this is likely the case for the CD44 gene [66]. The chromatin remodeling factor SWI/SNF is known to interact with pol II, splicing factors and spliceosome-associated proteins. SWI/SNF favors the inclusion of a block of consecutive alternative exons in the middle of *CD44*, by interacting with complexes containing U1 and U5 snRNPs and the nuclear RNA binding protein Sam68. These multimolecular complexes promote stalling of pol II at the central block of alternative exons, favoring their inclusion into mature mRNA. Most interestingly, SWI/SNF causes a

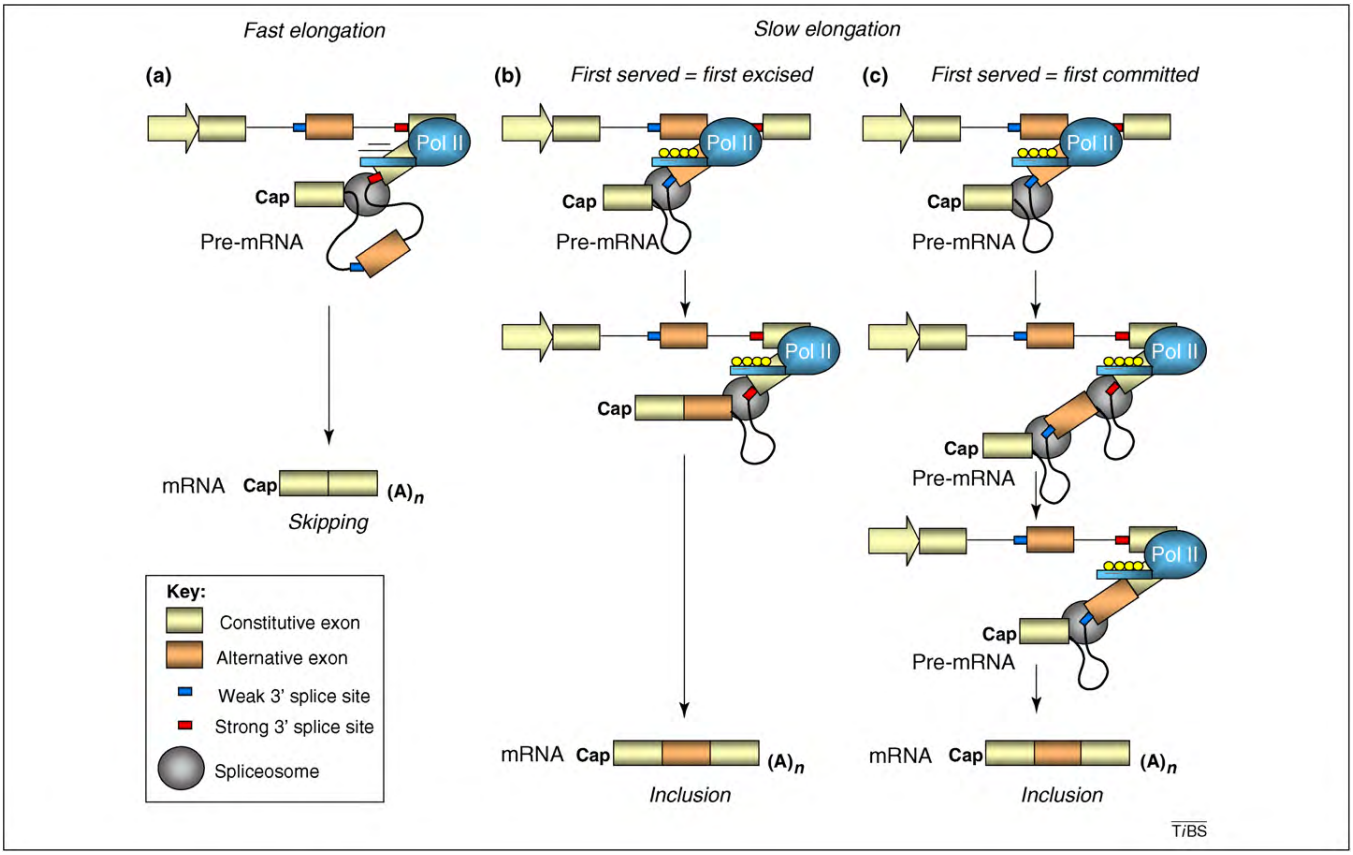

Figure 3. Kinetic coupling model for the regulation of alternative splicing by pol II elongation. In this particular example, slow elongation is caused by the CTD hyperphosphorylation (yellow circles) that follows UV-triggered DNA damage [79]. The 3' splice site by the alternative cassette exon (blue) is weaker than the 3' splice site of the downstream intron (red). High elongation rates **(a)** favor skipping, whereas low transcriptional elongation rates **(b)** and **(c)** favor exon inclusion. **(b)** and **(c)** depict two alternative pathways for the ''first come, first served'' mechanism of splice site selection leading to higher exon inclusion. **(b)** Slow elongation causes preferential excision of the upstream intron (first served = first excised). **(c)** Slow elongation causes commitment to inclusion of the alternative exon via recruitment of splicing factors (first served = first committed) independent of the relative order of intron removal [64].

switch of the pol II phosphorylation status from phosphoserine 2 (P-Ser2) to phosphoserine 5 (P-Ser5) at the internal stalling region. Such a change in CTD phosphorylation to that typical of promoters could generate internal "road blocks" to elongation, implying that the elongation rates of pol II can be modulated locally. A similar mechanism in which splicing is controlled by pol II stalling was recently supported by findings that membrane depolarization of neural cells affects neural cell adhesion molecule (*NCAM*) AS by changing intragenic histone acetylation patterns, and therefore the distribution of pol II, in an internal region surrounding the alternative exon [67]. Regardless of the model or the involvement of CTD, there is much documentation of the coupling between transcription and AS [65]. Such coupling involves effects of promoter identity and occupation by transcription factors [5,68–72], transcriptional activators and coactivators [73,74] and a panoply of proteins that either naturally function in both transcription and AS, or affect AS only when tethered to promoters or transcription components (reviewed in Ref. 65).

CTD phosphorylation and kinetic coupling

Although CTD phosphorylations of Ser2 and Ser5 are, by far, the most studied PTMs of the CTD, we still do not know much about their patterns (Box 1). Phosphorylation of Ser5 by cyclin-dependent kinase 7 (CDK7), a component of the basal transcription factor TFIIH, is linked to transcriptional initiation, whereas phosphorylation of Ser2 by CDK9, a component of the elongation factor P-TEFb, is associated with transcriptional elongation [35]. However, given that there are 46 Ser2 residues and 51 Ser5 residues in mammalian CTDs, this "rule" might be an oversimplification. Recent results show that UV irradiation affects cotranscriptional AS through pol II CTD hyperphosphorylation, at Ser2 and Ser5, and a subsequent inhibition of transcriptional elongation, in accordance with the kinetic model of coupling (Figure 3) [75]. The same effect on AS was obtained by the use of pol II CTD phosphomimetic mutants with glutamic acid at either position 2 (Glu2) or 5 (Glu5) and, consistent with a CTD-dependent mechanism, the UV effect on AS was prevented when Ser2 and Ser5 were each replaced by alanine. In an opposite scenario, at least for CTD hyperphosphorylation, treatment with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) inhibits basal and UV-induced hyperphosphorylation, and transcriptional elongation [76]. DRB is an inhibitor of the CTD kinase CDK9, a subunit of the elongation factor P-TEFb mentioned above. This finding might suggest that the CTD has at least two kinds of hyperphosphorylation states, both of which can be inhibited by DRB and both of which can be identified as pol IIO. It is conceivable that the CTD hyperphosphorylation state that inhibits elongation, such as that provoked by treatment with UV and mimicked in the Glu2/Glu5 mutants, differs from the CTD hyperphosphorylation state of non-irradiated cells, characteristic of an elongating pol II. The fact that the Glu2 and Glu5 mutations are present in all CTD repeats suggests that a homogeneous pattern of hyperphosphorylation could be the cause of lower elongation rates. Inhibition of elongation by either DRB [74,75] or UV [75] affects AS in the same

Box 1. Defining CTD phosphorylation patterns: still a molecular headache

A western blot analysis of pol II reveals mainly two isoforms: a hypophosphorylated (pol IIA) or hyperphosphorylated CTD (pol IIO) (Figure I). Variants lacking a unique 10 amino acid sequence (ISPDDSDEEN) located at the C terminus of heptad 52 suffer proteolytic degradation of the entire CTD *in vivo*, giving rise to a third isoform called pol IIB [88]. The pol IIA and pol IIO isoforms refer to their electrophoretic mobility on SDS-PAGE, but these gels do not provide any information about whether the pol IIO isoform contains an homogeneous population of molecules because the patterns of phosphorylation on individual pol II CTDs can vary widely while still migrating similarly in SDS-PAGE. For instance, differential phosphorylation of serine 2 (Ser2) versus serine 5 (Ser5) and/or an uneven versus an even distribution of the same number of phosphate groups among the 52 CTD repeats would result in pol IIO bands with identical mobility. Moreover, the specificity of the widely used and commercially available antibodies designed to recognize P-Ser5 and P-Ser2 epitopes in the CTD has been, and remains, a subject of great debate [30], pushing some laboratories to make and validate their own anti-phospho-CTD antibodies. Thus, we are dealing with a situation in which unequivocal assignment of CTD hyperphosphorylation patterns cannot be achieved, which undoubtedly hinders the in-depth understanding of the mechanisms of CTD-dependent RNA processing. For instance, different pre-mRNAs might have different dependence on the number of the CTD repeats for efficient processing [89]; indeed, the C-terminal half (from heptad 27 to the end) of the CTD supports all three processing reactions, whereas the N-terminal half of the CTD supports only capping [90]. Because both CTD halves differ in composition, with the C-terminal half being richer in degenerate heptads, these results, together with the notion that the number of repeats increases with organism complexity [91], are in agreement with the idea that the different CTD repeats are not functionally equivalent. In this respect, it is reasonable to hypothesize that PTMs along the CTD repeats are not equivalent.

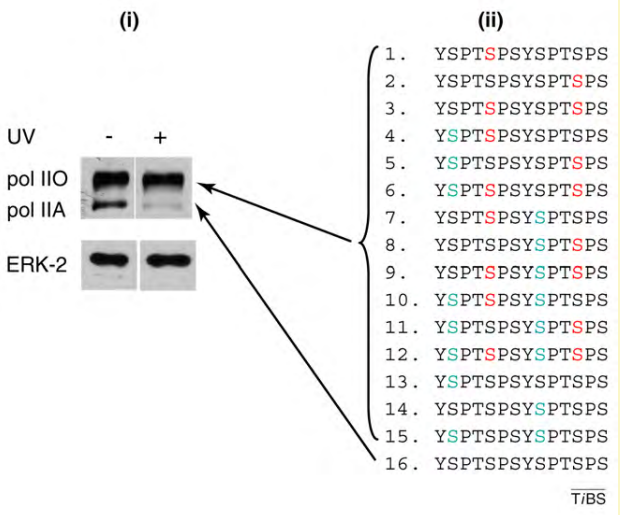

Figure I. Difficulties in discriminating phosphorylation patterns. (i) Western blot with permission of Cell Press of human RPB1 using an antibody directed to the non-repetitive N-terminus of the molecule. Protein extracts were obtained from hepatoma Hep3B cells, non-irradiated (–) or irradiated (+) with 254 nm wavelength UV light at 20 J/m^2. The antibody recognizes both the hyper- (pol IIO) and hypo- (pol IIA) phosphorylated forms of RPB1. UV irradiation causes an increase in the relative abundance of pol IIO; an antibody to ERK2 was used as a control. (ii) Sixteen (4^2) possible combinations of phosphorylation at Ser2 and Ser5 of a CTD hypothetically formed by only two heptad repeats. For a real CTD, the maximum number of variants would be approximately 4^{52}. This extremely simplified example illustrates how little information about phosphorylation patterns can be extracted from this kind of widely used western blot. On the one hand, for the 16 possible variants, only variant 16 unequivocally identifies pol IIA, whereas any combination or mix of variants 1–15 can be assigned to the pol IIO band. By contrast, an antibody specific for P-Ser5 (red) would detect variants 1–12, whereas an antibody specific to P-Ser2 (light blue) would detect variants 4–15; variants 4–12 would be detected by both antibodies.

way in Hep3B cells, whereas in HCT116 cells DRB has little effect *per se* on EDI inclusion, but abrogates its stimulation by UV [75]. These results imply that additional cell-type-specific factors influence how CTD phosphorylation affects AS, a notion that is reminiscent of the recruitment model of coupling, and again favors a unified view of the coupling mechanisms. Together with results showing that Ser2 phosphorylation is not necessary for transcriptional elongation in some genes [77], it is clear that the assignment of Ser5 and Ser2 phosphorylation as marks of initiation and elongation, respectively, might not be the rule, at least for all genes or situations.

The CTD undergoes other PTMs. For instance, there is evidence for Tyr1 phosphorylation by the c-ABL kinase [78] and Ser7 phosphorylation by CDK7 [79–81]. The latter can affect transcription, but its effects on pre-mRNA processing remain unexplored. Future research in this direction will face the difficult challenge of dealing with an enormous number of possible CTD configurations, potentially affecting gene-specific or even exon-specific splicing events.

Concluding remarks and future perspectives

An updated model for the coupling of transcription and AS is clearly emerging. In this new scenario, chromatin architecture and its dynamic nature will have a crucial role in dictating the fate of splice site decisions. The recently described correlations between nucleosome deposition, together with histone tail modifications and exon definition [82–84], point toward a direction where much of the future research will be focused. In fact, recent reports demonstrate that changes in chromatin organization, triggered by different means, can impact on AS regulation [67,85,86]. In this context, the pol II CTD might also play a role, given its known set of interactions with chromatin modification factors [87]. However, technical challenges remain in characterizing the CTD PTM patterns and their possible implications in mediating coupling through chromatin. In this respect, the biochemical and structural characterization of different CTD configurations and how this relates to the interactions between the CTD and chromatin/splicing factors remains one of the biggest challenges in the field. From a more functional point of view, future directions might involve use of knock-in cells and mice where phosphomimetic CTD mutants or the slow version of pol II replace the WT gene in order to study global and cell-specific changes in AS. These studies could help to identify transcriptionally regulated AS events that control fundamental processes such as cell survival, apoptosis, differentiation and malignant transformation. Overall, the wide variety of discoveries made since 1997 forces us to think in stereo, with one ear listening to the channel of transcription and the other to the channel of splicing, if we want to listen to the full orchestra.

Acknowledgments

We thank B. Graveley, D. Bentley and D. Reinberg for useful discussions. This work was supported by grants to A.R.K. from the Agencia Nacional de Promoción de Ciencia y Tecnología of Argentina (ANPCYT), the University of Buenos Aires and the European Alternative Splicing Network (EURASNET). M.J.M. is a recipient of a post-doctoral fellowship and A.R.K. is a career investigator from the Consejo Nacional de Investigaciones Científicas y Técnicas of Argentina (CONICET). A.R.K is an international research scholar of the Howard Hughes Medical Institute.

References

1 Corden, J.L. *et al.* (1985) A unique structure at the carboxyl terminus of the largest subunit of eukaryotic RNA polymerase II. *Proc. Natl. Acad. Sci. U. S. A.* 82, 7934–7938
2 Gerber, H.P. *et al.* (1995) RNA polymerase II C-terminal domain required for enhancer-driven transcription. *Nature* 374, 660–662
3 McCracken, S. *et al.* (1997) The C-terminal domain of RNA polymerase II couples mRNA processing to transcription. *Nature* 385, 357–361
4 McCracken, S. *et al.* (1997) 5′-Capping enzymes are targeted to pre-mRNA by binding to the phosphorylated carboxy-terminal domain of RNA polymerase II. *Genes Dev.* 11, 3306–3318
5 Cramer, P. *et al.* (1997) Functional association between promoter structure and transcript alternative splicing. *Proc. Natl. Acad. Sci. U. S. A.* 94, 11456–11460
6 Kim, E. *et al.* (2007) Different levels of alternative splicing among eukaryotes. *Nucleic Acids Res.* 35, 125–131
7 Pan, Q. *et al.* (2008) Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. *Nat. Genet.* 40, 1413–1415
8 Wang, E.T. *et al.* (2008) Alternative isoform regulation in human tissue transcriptomes. *Nature* 456, 470–476
9 Orengo, J.P. and Cooper, T.A. (2007) Alternative splicing in disease. *Adv. Exp. Med. Biol.* 623, 212–223
10 Caceres, J.F. and Kornblihtt, A.R. (2002) Alternative splicing: multiple control mechanisms and involvement in human disease. *Trends Genet.* 18, 186–193
11 Cartegni, L. *et al.* (2002) Listening to silence and understanding nonsense: exonic mutations that affect splicing. *Nat. Rev. Genet.* 3, 285–298
12 Faustino, N.A. and Cooper, T.A. (2003) Pre-mRNA splicing and human disease. *Genes Dev.* 17, 419–437
13 Pagani, F. and Baralle, F.E. (2004) Genomic variants in exons and introns: identifying the splicing spoilers. *Nat. Rev. Genet.* 5, 389–396
14 David, C.J. *et al.* (2010) hnRNP proteins controlled by c-Myc deregulate pyruvate kinase mRNA splicing in cancer. *Nature* 463, 364–368 zzz
15 Ghigna, C. *et al.* (2005) Cell motility is controlled by SF2/ASF through alternative splicing of the Ron protooncogene. *Mol. Cell* 20, 881–890
16 Karni, R. *et al.* (2007) The gene encoding the splicing factor SF2/ASF is a proto-oncogene. *Nat. Struct. Mol. Biol.* 14, 185–193
17 Srebrow, A. and Kornblihtt, A.R. (2006) The connection between splicing and cancer. *J. Cell Sci.* 119, 2635–2641
18 Ding, J.H. *et al.* (2004) Dilated cardiomyopathy caused by tissue-specific ablation of SC35 in the heart. *EMBO J.* 23, 885–896
19 Xu, X. *et al.* (2005) ASF/SF2-regulated CaMKIIdelta alternative splicing temporally reprograms excitation-contraction coupling in cardiac muscle. *Cell* 120, 59–72
20 Feng, Y. *et al.* (2009) SRp38 regulates alternative splicing and is required for Ca(2+) handling in the embryonic heart. *Dev. Cell* 16, 528–538
21 Graveley, B.R. (2009) Alternative splicing: regulation without regulators. *Nat. Struct. Mol. Biol.* 16, 13–15
22 Xu, Y.X. and Manley, J.L. (2004) Pinning down transcription: regulation of RNA polymerase II activity during the cell cycle. *Cell Cycle* 3, 432–435
23 Xu, Y.X. and Manley, J.L. (2007) Pin1 modulates RNA polymerase II activity during the transcription cycle. *Genes Dev.* 21, 2950–2962
24 Kelly, W.G. *et al.* (1993) RNA polymerase II is a glycoprotein. Modification of the COOH-terminal domain by O-GlcNAc. *J. Biol. Chem.* 268, 10416–10424
25 de Almeida, S.F. and Carmo-Fonseca, M. (2008) The CTD role in cotranscriptional RNA processing and surveillance. *FEBS Lett.* 582, 1971–1976
26 Egloff, S. and Murphy, S. (2008) Cracking the RNA polymerase II CTD code. *Trends Genet.* 24, 280–288
27 Perales, R. and Bentley, D. (2009) "Cotranscriptionality": the transcription elongation complex as a nexus for nuclear transactions. *Mol. Cell* 36, 178–191
28 de la Mata, M. and Kornblihtt, A.R. (2006) RNA polymerase II C-terminal domain mediates regulation of alternative splicing by SRp20. *Nat. Struct. Mol. Biol.* 13, 973–980

29 Meininghaus, M. *et al.* (2000) Conditional expression of RNA polymerase II in mammalian cells. Deletion of the carboxyl-terminal domain of the large subunit affects early steps in transcription. *J. Biol. Chem.* 275, 24375–24382
30 Phatnani, H.P. and Greenleaf, A.L. (2006) Phosphorylation and functions of the RNA polymerase II CTD. *Genes Dev.* 20, 2922–2936
31 Yoh, S.M. *et al.* (2008) The Iws1:Spt6:CTD complex controls cotranscriptional mRNA biosynthesis and HYPB/Setd2-mediated histone H3K36 methylation. *Genes Dev.* 22, 3422–3434
32 Cho, H. *et al.* (1998) A human RNA polymerase II complex containing factors that modify chromatin structure. *Mol. Cell Biol.* 18, 5355–5363
33 Bentley, D.L. (2005) Rules of engagement: co-transcriptional recruitment of pre-mRNA processing factors. *Curr. Opin. Cell Biol.* 17, 251–256
34 Shuman, S. (2001) Structure, mechanism, and evolution of the mRNA capping apparatus. *Prog. Nucleic Acid Res. Mol. Biol.* 66, 1–40
35 Buratowski, S. (2009) Progression through the RNA polymerase II CTD cycle. *Mol. Cell* 36, 541–546
36 Ahn, S.H. *et al.* (2004) Phosphorylation of serine 2 within the RNA polymerase II C-terminal domain couples transcription and 3' end processing. *Mol. Cell* 13, 67–76
37 Meinhart, A. and Cramer, P. (2004) Recognition of RNA polymerase II carboxy-terminal domain by 3'-RNA-processing factors. *Nature* 430, 223–226
38 Misteli, T. and Spector, D.L. (1999) RNA polymerase II targets pre-mRNA splicing factors to transcription sites in vivo. *Mol. Cell* 3, 697–705
39 Zeng, C. and Berget, S.M. (2000) Participation of the C-terminal domain of RNA polymerase II in exon definition during pre-mRNA splicing. *Mol. Cell Biol.* 20, 8290–8301
40 Hirose, Y. *et al.* (1999) Phosphorylated RNA polymerase II stimulates pre-mRNA splicing. *Genes Dev.* 13, 1234–1239
41 Beyer, A.L. and Osheim, Y.N. (1988) Splice site selection, rate of splicing, and alternative splicing on nascent transcripts. *Genes Dev.* 2, 754–765
42 Bird, G. *et al.* (2004) RNA polymerase II carboxy-terminal domain phosphorylation is required for cotranscriptional pre-mRNA splicing and 3'-end formation. *Mol. Cell Biol.* 24, 8963–8969
43 Cramer, P. *et al.* (2001) Structural basis of transcription: RNA polymerase II at 2.8 angstrom resolution. *Science* 292, 1863–1876
44 Natalizio, B.J. *et al.* (2009) The carboxyl-terminal domain of RNA polymerase II is not sufficient to enhance the efficiency of pre-mRNA capping or splicing in the context of a different polymerase. *J. Biol. Chem.* 284, 8692–8702
45 Shepard, P.J. and Hertel, K.J. (2009) The SR protein family. *Genome Biol.* 10, 242
46 Eperon, L.P. *et al.* (1988) Effects of RNA secondary structure on alternative splicing of pre-mRNA: is folding limited to a region behind the transcribing RNA polymerase? *Cell* 54, 393–401
47 Lazarev, D. and Manley, J.L. (2007) Concurrent splicing and transcription are not sufficient to enhance splicing efficiency. *RNA* 13, 1546–1557
48 Das, R. *et al.* (2006) Functional coupling of RNAP II transcription to spliceosome assembly. *Genes Dev.* 20, 1100–1109
49 Gornemann, J. *et al.* (2005) Cotranscriptional spliceosome assembly occurs in a stepwise fashion and requires the cap binding complex. *Mol. Cell* 19, 53–63
50 Kornblihtt, A.R. *et al.* (2004) Multiple links between transcription and splicing. *RNA* 10, 1489–1498
51 Listerman, I. *et al.* (2006) Cotranscriptional coupling of splicing factor recruitment and precursor messenger RNA splicing in mammalian cells. *Nat. Struct. Mol. Biol.* 13, 815–822
52 Pandya-Jones, A. and Black, D.L. (2009) Co-transcriptional splicing of constitutive and alternative exons. *RNA* 15, 1896–1908
53 Singh, J. and Padgett, R.A. (2009) Rates of in situ transcription and splicing in large human genes. *Nat. Struct. Mol. Biol.* 16, 1128–1133
54 Wetterberg, I. *et al.* (2001) In situ transcription and splicing in the Balbiani ring 3 gene. *EMBO J.* 20, 2564–2574
55 Hicks, M.J. *et al.* (2006) Linking splicing to Pol II transcription stabilizes pre-mRNAs and influences splicing patterns. *PLoS Biol.* 4, e147
56 Das, R. *et al.* (2007) SR proteins function in coupling RNAP II transcription to pre-mRNA splicing. *Mol. Cell* 26, 867–881
57 Lacadie, S.A. *et al.* (2006) In vivo commitment to yeast cotranscriptional splicing is sensitive to transcription elongation mutants. *Genes Dev.* 20, 2055–2066
58 Listerman, I. *et al.* (2006) Co-transcriptional coupling of splicing factor recruitment and pre-mRNA splicing in mammalian cells. *Nat. Struct. Mol. Biol.* 13, 815–822
59 Sapra, A.K. *et al.* (2009) SR protein family members display diverse activities in the formation of nascent and mature mRNPs in vivo. *Mol. Cell* 34, 179–190
60 Aguilera, A. and Gomez-Gonzalez, B. (2008) Genome instability: a mechanistic view of its causes and consequences. *Nat. Rev. Genet.* 9, 204–217
61 Li, X. and Manley, J.L. (2005) Inactivation of the SR protein splicing factor ASF/SF2 results in genomic instability. *Cell* 122, 365–378
62 de la Mata, M. *et al.* (2003) A slow RNA polymerase II affects alternative splicing in vivo. *Mol. Cell* 12, 525–532
63 Kadener, S. *et al.* (2001) Antagonistic effects of T-Ag and VP16 reveal a role for RNA pol II elongation on alternative splicing. *EMBO J.* 20, 5759–5768
64 de la Mata, M. *et al.* (2010) First come, first served revisited: factors affecting the same alternative splicing event have different effects on the relative rates of intron removal. *RNA* 16, 904–912
65 Kornblihtt, A.R. (2007) Coupling transcription and alternative splicing. *Adv. Exp. Med. Biol.* 623, 175–189
66 Batsche, E. *et al.* (2006) The human SWI/SNF subunit Brm is a regulator of alternative splicing. *Nat. Struct. Mol. Biol.* 13, 22–29
67 Schor, I.E. *et al.* (2009) Neuronal cell depolarization induces intragenic chromatin modifications affecting NCAM alternative splicing. *Proc. Natl. Acad. Sci. U. S. A.* 106, 4325–4330
68 Auboeuf, D. *et al.* (2002) Coordinate regulation of transcription and splicing by steroid receptor coregulators. *Science* 298, 416–419
69 Cramer, P. *et al.* (1999) Coupling of transcription with alternative splicing: RNA pol II promoters modulate SF2/ASF and 9G8 effects on an exonic splicing enhancer. *Mol. Cell* 4, 251–258
70 Kornblihtt, A.R. (2005) Promoter usage and alternative splicing. *Curr. Opin. Cell Biol.* 17, 262–268
71 Pagani, F. *et al.* (2003) Promoter architecture modulates CFTR exon 9 skipping. *J. Biol. Chem.* 278, 1511–1517
72 Robson-Dixon, N.D. and Garcia-Blanco, M.A. (2004) MAZ elements alter transcription elongation and silencing of the fibroblast growth factor receptor 2 exon IIIb. *J. Biol. Chem.* 279, 29075–29084
73 Auboeuf, D. *et al.* (2004) CoAA, a nuclear receptor coactivator protein at the interface of transcriptional coactivation and RNA splicing. *Mol. Cell Biol.* 24, 442–453
74 Nogues, G. *et al.* (2002) Transcriptional activators differ in their abilities to control alternative splicing. *J. Biol. Chem.* 277, 43110–43114
75 Munoz, M.J. *et al.* (2009) DNA damage regulates alternative splicing through inhibition of RNA polymerase II elongation. *Cell* 137, 708–720
76 Darzacq, X. *et al.* (2007) In vivo dynamics of RNA polymerase II transcription. *Nat. Struct. Mol. Biol.* 14, 796–806
77 Gomes, N.P. *et al.* (2006) Gene-specific requirement for P-TEFb activity and RNA polymerase II phosphorylation within the p53 transcriptional program. *Genes Dev.* 20, 601–612
78 Baskaran, R. *et al.* (1999) Nuclear c-Abl is a COOH-terminal repeated domain (CTD)-tyrosine (CTD)-tyrosine kinase-specific for the mammalian RNA polymerase II: possible role in transcription elongation. *Cell Growth Differ.* 10, 387–396
79 Akhtar, M.S. *et al.* (2009) TFIIH kinase places bivalent marks on the carboxy-terminal domain of RNA polymerase II. *Mol. Cell* 34, 387–393
80 Chapman, R.D. *et al.* (2007) Transcribing RNA polymerase II is phosphorylated at CTD residue serine-7. *Science* 318, 1780–1782
81 Egloff, S. *et al.* (2007) Serine-7 of the RNA polymerase II CTD is specifically required for snRNA gene expression. *Science* 318, 1777–1779
82 Kolasinska-Zwierz, P. *et al.* (2009) Differential chromatin marking of introns and expressed exons by H3K36me3. *Nat. Genet.* 41, 376–381
83 Schwartz, S. *et al.* (2009) Chromatin organization marks exon-intron structure. *Nat. Struct. Mol. Biol.* 16, 990–995
84 Tilgner, H. *et al.* (2009) Nucleosome positioning as a determinant of exon recognition. *Nat. Struct. Mol. Biol.* 16, 996–1001

85 Allo, M. *et al.* (2009) Control of alternative splicing through siRNA-mediated transcriptional gene silencing. *Nat. Struct. Mol. Biol.* 16, 717–724
86 Luco, R.F. *et al.* (2010) Regulation of alternative splicing by histone modifications. *Science* 327, 996–1000
87 Campos, E.L. and Reinberg, D. (2009) Histones: annotating chromatin. *Annu. Rev. Genet.* 43, 559–599
88 Chapman, R.D. *et al.* (2004) The last CTD repeat of the mammalian RNA polymerase II large subunit is important for its stability. *Nucleic Acids Res.* 32, 35–44
89 Rosonina, E. and Blencowe, B.J. (2004) Analysis of the requirement for RNA polymerase II CTD heptapeptide repeats in pre-mRNA splicing and 3'-end cleavage. *RNA* 10, 581–589
90 Chapman, R.D. *et al.* (2008) Molecular evolution of the RNA polymerase II CTD. *Trends Genet.* 24, 289–296
91 Fong, N. and Bentley, D.L. (2001) Capping, splicing, and 3' processing are independently stimulated by RNA polymerase II: different functions for different segments of the CTD. *Genes Dev.* 15, 1783–1795